

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 7/20, 7/26, 7/06 A61K 37/24, 37/43		A1	(11) International Publication Number: WO 91/18016 (43) International Publication Date: 28 November 1991 (28.11.91)
(21) International Application Number: PCT/EP91/00727 (22) International Filing Date: 17 April 1991 (17.04.91) (30) Priority data: 20273 A/90 11 May 1990 (11.05.90) IT (71)(72) Applicant and Inventor: DEGHENGHI, Romano [IT/CH]; Chesaux Dessus B1, CH-1264 S.-Cergue (CH). (74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8, I-20122 Milano (IT). (81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: BIOLOGICALLY ACTIVE PEPTIDES CONTAINING D-2-ALKYLTRYPTOPHAN			
(57) Abstract <p>Peptides containing at least one D-2-alkyltryptophan residue in their amino acid chain and having pharmacological activity similar to that of analogous peptides containing natural unsubstituted D-tryptophan residues in place of at least one D-2-alkyltryptophan. These new peptides are more resistant to oxidative degradation which usually takes place, for example, in the presence of reactive radicals or during high energy sterilization than unsubstituted tryptophan containing peptides.</p>			

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

BIOLOGICALLY ACTIVE PEPTIDES
CONTAINING D-2-ALKYLTRYPTOPHAN

Technical Field

The present invention relates to the field of biologically active peptides. Specifically, this invention relates to biologically active peptides containing the amino acid D-Tryptophan (D-Trp).

Background Art

It is well known that the incorporation or substitution of a D-Tryptophan residue into a biologically active peptide chain enhances the activity of that chain. Furthermore, such incorporation or substitution will prolong the biological activity. The prolonged and enhanced effectiveness of such peptides probably relates the increased resistance to degradation by peptidases.

Examples of D-Tryptophan containing peptides are the LHRH agonists as described by D.H. Coy et al., Journal of Medical Chemistry, volume 19, page 423 (1976), W. Koenig et al., Peptide Chemistry (1987), T. Shiba and S. Sakakibara (eds.), Osaka, Protein Research Foundation, Osaka (1988), page 591, B.J.A. Furr et al., Journal of Endocrinol. Invest., volume 11, page 535 (1988). Examples of D-Tryptophan containing somatostatin analogs, such as the peptides octreotide and vapreotide are disclosed by R. Deghenghi, Biomedicine and Pharmacotherapy, volume 42, page 585 (1988). Another example of a D-Tryptophan containing peptide are the synthetic antagonists of Substance P as disclosed by D. Regoli et al., European Journal of Pharmacology, volume 99, page 193, (1984), and GHRP-6 described by C.Y. Bowers et al., Endocrinology, volume 114, page 1537, (1984).

Peptides containing Tryptophan have been subject to degradation due to the "Kynurenine pathway". In this pathway, the enzyme Tryptophan pyrrolase (i.e., indolamine 2,3-dioxygenase) degrades the pyrrole ring of Tryptophan. Kynurenine and other breakdown products

- 2 -

are generated by this degradation. Some of the breakdown products have been shown to be toxic when present in elevated concentrations as reported by R.M. Silver et al., The New England Journal of Medicine,
5 volume 322, page 874, (1990).

D-Tryptophan containing peptides are subject to degradation by oxygen and other reactive radicals as reported by R. Geiger and W. Koenig, "The Peptides," Academic Press, volume 3, page 82, New York (1981).
10 The D-Tryptophan in the peptide chain may react with active or activated groups when peptides are formulated in certain controlled delivery pharmaceutical compositions, such as those based on polylactic-polyglycolic acid polymers. Such
15 degradation is thought to be facilitated by either heat or by the presence of catalysts. It is also possible that radiolysis products formed during ionizing sterilization of these pharmaceutical compositions may facilitate the breakdown of D-Tryptophan. Clearly, the
20 breakdown of D-Tryptophan, and the concomitant breakdown of the pharmaceutical compound containing D-Tryptophan is an undesirable effect.

What is needed is a derivative of D-Tryptophan which retains the prolonged and increased
25 biological activity discussed above, while resisting degradation by indolamine dioxygenase, oxygen, or other reactive radicals. It is of course essential that such a derivative of D-Tryptophan would maintain biological activity as compared to natural D-Tryptophan containing
30 bioactive peptides.

The terms "biological effect" or "pharmacological effect" as used in the present disclosure refer to the qualitative effect that a bioactive peptide has upon living tissue. As an
35 example, LHRH, luteinizing hormone releasing hormone, has the biological effect of causing cells of the anterior pituitary gland to release luteinizing

- 3 -

hormone. In contrast, the term "potency" is used in its conventional sense to refer to the degree and duration of the bioactivity of a given peptide.

Utilizing these terms as defined above, what is needed is a Tryptophan containing bioactive peptide which is resistant to oxidative degradation and reactive radical attack while maintaining the same biological activity and a similar or greater potency than the presently available analogous peptides provide.

Summary of Invention

Now in accordance with the present invention, a derivative of D-Tryptophan has been discovered which imparts to a biologically active peptide incorporating the derivative improved resistance to oxidative breakdown reactions of the Tryptophan derivative, while maintaining the biological activity and pharmacological effect exhibited by peptides incorporating unaltered D-Tryptophan. Specifically, the present invention relates to biologically active peptides incorporating at least one D-Tryptophan in which a lower alkyl group, preferably an alkyl group containing 1 to 3 carbons, is substituted in the 2 position. Peptides incorporating such substituted D-Tryptophans are more stable in the presence of reactive radicals, or when pharmaceuticals containing such peptides are exposed to ionizing radiation.

30 Detailed Description

Biologically active peptides in accordance with the present invention are characterized by the following formula:

35

A-D-2-ALK-TRP-B

- 4 -

where alk is a lower alkyl group, preferably comprising 1 to 3 carbons. A is SEQUENCE ID NO:1, wherein the Glutamic Acid residue at SEQUENCE position 1 is Pyro-Glu; D-Phe-Cys-Phe; SEQUENCE ID NO:2 wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Proline residue at SEQUENCE position 4 is D-Proline and the Tryptophan residues at SEQUENCE positions 7 and 9 are D-Tryptophan; Arg-D-Trp-N-methyl-Phe; or His; and B is SEQUENCE ID NO:3 wherein the Glycine residue at sequence position 4 is Gly-NH₂; Leu-Arg-Pro-NHCH₂CH₃; Lys-Thr-Cys-NHCH(CH₂OH)CHOH(CH₃); SEQUENCE ID NO:4 wherein the Tryptophan residue at SEQUENCE position 4 is Trp-NH₂; Met-NH₂; Leu-Met-NH₂; SEQUENCE ID NO:5 wherein the Phenylalanine residue at SEQUENCE position 3 is D-Phe and the Lysine residue at SEQUENCE position 4 is Lys-NH₂; provided that when A is SEQUENCE ID NO:1, B is SEQUENCE ID NO:3 wherein the Glycine residue at SEQUENCE position 4 is Gly-NH₂ or Leu-Arg-Pro-NHCH₂CH₃; when A is D-Phe-Cys-Phe, B is Lys-Thr-Cys-NHCH-(CH₂OH)CHOH(CH₃) wherein the cysteine units are bound to each other by a cyclic disulphide; when A is D-Phe-Cys-Tyr, B is SEQUENCE ID NO:4 wherein the cysteine units are bound to each other by a cyclic disulfide and the Tryptophan residue at SEQUENCE position 4 is Trp-NH₂; when A is SEQUENCE ID NO:6 wherein the Proline residue in SEQUENCE position 1 is D-Pro and the Tryptophan residues at SEQUENCE positions 4 and 6 are D-Trp, B is Met-NH₂; when A is Arg-D-Trp-n-methyl-Phe, B is Leu-Met-NH₂; or when A is His, B is SEQUENCE ID NO:5 wherein the Phenylalanine residue at SEQUENCE position 3 is D-Phe and the Lysine residue at SEQUENCE position 4 is Lys-NH₂.

The following compounds are biologically active peptides of the present invention which contain at least one C-2 substituted D-Tryptophan according to the present invention.

- 5 -

A. SEQUENCE ID NO:7 wherein the Glutamic acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Glycine residue at SEQUENCE
5 position 10 is Pro-Gly-NH₂.

B. SEQUENCE ID NO:8 wherein the Glutamic Acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Proline residue at SEQUENCE
10 position 9 is Pro-NHCH₂CH₃.

C. SEQUENCE ID NO:9 wherein the Proline residue at SEQUENCE position 1 is D-Pro, the Tryptophan residue at SEQUENCE position 4 is D-Trp, the Tryptophan residue at SEQUENCE position 6 is D-Trp, the Tryptophan
15 residue at SEQUENCE position 7 is D-2-ethyl-Trp, and the Methionine residue at SEQUENCE position 8 is Met-NH₂.

D. SEQUENCE ID NO:10 wherein the Tryptophan residue at SEQUENCE position 2 is D-Trp, the
20 Phenylalanine residue at SEQUENCE position 3 is N-methyl-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-isopropyl-Trp, and the Methionine residue at SEQUENCE position 6 is Met-NH₂.

E. SEQUENCE NO:11 (and its cyclic oxidation
25 product) wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-methyl-Trp and the Cysteine residue at SEQUENCE position 7 is Cys-NHCH(CH₂OH)CHOHCH₃.

F. SEQUENCE ID NO:12 (and its cyclic
30 oxidation product) wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-methyl-Trp, and the Tryptophan residue at SEQUENCE position 8 is Trp-NH₂.

G. SEQUENCE ID NO:13 wherein the Tryptophan
35 residue at SEQUENCE position 2 is D-2-methyl Trp, the

- 6 -

Phenylalanine residue at SEQUENCE position 5 is D-Phe, and the Lysine residue at position 6 is Lys-NH₂.

Compounds A and B are analogs of the natural peptide SEQUENCE ID NO:14 wherein the Glutamic acid residue at SEQUENCE position 1 is Pyro-Glu and the Glycine residue at position 10 is Gly-NH₂. SEQUENCE ID NO:14 is luteinizing hormone releasing hormone (LH-RH), a neurohumoral hormone produced in the hypothalamus which stimulates the secretion of the LH luteinizing hormone by the anterior pituitary gland. Compounds A and B pertain therefore to the class of LHRH agonists and are also defined respectively as follows:
[D-2-methyl-Trp⁶]LHRH and [Des-Gly¹⁰-D-2-methyl-Trp⁶-Pro-ethylamide⁹]LHRH.

Compounds C and D are antagonists of substance P. Substance P is a neurotransmitter used by sensory neurons that convey responses of pain or other noxious stimuli to the central nervous system. Compounds C and D therefore have analgesic and anti-inflammatory effects. Peptides E and F are analogs (agonists) of somatostatin and as such show antisecretory and antitumoral activity. Peptide G is an analog of GHRP (Growth Hormone Releasing Peptide) stimulating the release of growth hormone.

2-methyl-Tryptophan is known (cf. H.N. Rydon, J. Chem. Soc. 1948, 705) and the homologous alkylated derivatives are conveniently prepared from the corresponding 2-alkyl indoles by well known methods (cf. J.P. Li et al., Synthesis (1), 73, 1988). The resolution of the racemic Tryptophan derivatives to give the D-enantiomers of the present invention can also be achieved by a variety of methods (cf. Amino Acids, Peptides and Proteins, Vol. 16, pages 18-20, The Royal Society of Chemistry, London, 1985). Both the solution phase or the solid phase method of peptide synthesis can be used to make the peptides of this invention, (cf. R. Geiger et al., "The Peptides",

- 7 -

Academic Press, New York 1981). If the solid phase method is used, peptide synthesizers such as the Applied Biosystem 430A, Bioresearch Sam 9500 or the Beckman Model 990 are preferably used.

- 5 According to this methodology, the first amino acid is linked to the benzhydrylamine resin and the remaining protected amino acids are then coupled in a step wise manner using the standard procedures recommended by the manufacturers of the synthesizers.
- 10 For instance, amino acid couplings are performed by using symmetrical anhydrides in the Applied Biosystems Synthesizer and diisopropylcarbodiimide in the Bioresearch or Beckman machines. The amino acid derivatives are protected by the tertiary
- 15 butoxy-carbonyl groups on the alpha-amino function during the synthesis. The functional groups present in the amino-acid in the side chain are previously protected, e.g. by acetyl(Ac), benzoyl (Bz), t-butyloxycarbonyl (Boc), benzyloxymethyl (Bom), benzyl
- 20 (Bzl), benzyloxycarbonyl (Z), formyl (For), p-nitro-phenyl ester (ONp), tosyl (Tos), etc. For instance, the functional groups of Histidine are protected by benzyloxymethyl (His(Bom)), tosyl (His(Tos)), the functional groups of Tryptophan by
- 25 formyl (Trp(For)), those of Serine by benzyl (Ser(Bzl)), those of Tyrosine by 2-Br-benzyloxycarbonyl (Tyr(2-Br-Z)), those of Arginine by tosyl (Arg(Tos)), those of Leucine by O-benzyl-p-tosyl (Leu(O-Bzl-p-Tos)), those of Proline by O-benzyl HCl (Pro(O-Bzl HCl)), those of Glycine by O-benzyl HCl (Gly (O-Bzl HCl)), those of Cysteine by 4-methyl-benzyl
- 30 (Cys(4-Me-Bzl)), those of Lysine by benzyloxycarbonyl (Lys(Z)), those of Threonine by benzyl-OH (Thr(Bzl-OH)), those of Valine by O-benzyl-tosyl
- 35 (Val(O-Bzl-p-Tos)), those of Glutamic Acid by O-benzyl (Glu(O-Bzl)), those of Methionine by P-nitrophenyl

- 8 -

ester (Me(Onp)), and those of Alanine by O-benzyl HCl (Ala(O-Bzl HCl)).

The Boc protective groups on the alpha-aminic function are removed at each stage by treatment with
5 60% trifluoroacetic acid ("TFA") in dichloromethane. Cleavage of Trp and Met containing peptides from the resin with simultaneous removal of all side-chain protecting groups is achieved as described by J.P. Tam et al., J. Am. Chem. Soc., Vol 105, page 6442 (1983).
10 The crude peptides after HF cleavage are purified on a Sephadex G-50 F column in 50% acetic acid or by preparative reverse phase HPLC using gradients of acetonitrile and water containing 0.1% trifluoroacetic acid.

15 The examples that follow are given for illustrative purposes only, but are not limitative of the present invention.

Example 1

20 SEQUENCE ID NO:7 wherein the Glutamic acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Glycine residue at SEQUENCE position 10 is Gly-NH₂.

25 The protective groups for the side chains are Tosyl (Tos) for Arginine and Histidine and Bromo-benzyloxycarbonyl (2-Br-Z) for Tyrosine. The benzhydrylamine resin (2.2g) (Bachem^R), was cross-linked at 1% with Proline and the apparatus used was a Beckman
30 Model 990. The amino acids protected by Boc (tert-butyloxycarbonyl) are coupled with dicyclohexylcarbodiimide. The Boc groups are removed by trifluoroacetic acid in methylene chloride.

The synthesis yielded 4.07 g of the
35 decapeptide-resin (98% of theoretical weight gain). Part of this resin (1.5 g) was stirred at 0° centigrade for 30 minutes with HF (24 ml) and anisole (8 ml). HF

- 9 -

was then removed as rapidly as possible (ca. 60 min) in vacuo and EtOAc was added to the thus obtained residue. Solid material was filtered, washed with EtOAc, dried, and extracted with 2 M AcOH. Lyophilization gave a white powder which was purified by gel filtration on a column (2.5 X 95 cm) of Sephadex G-25 (fine) by elution with 2 M AcOH. The eluate portion corresponding to the major peak was then dried and eluted further on a column (2.5 X 95 cm) of Sephadex G-25 (fine) previously equilibrated with the lower phase followed by the upper phase of the following biphasic solvent mixture n-BuOH-AcOH-H₂O (4:1:5). Elution with the upper phase gave a major peak and the peptide from this area was collected, concentrated to dryness, and lyophilized from dilute AcOH to give the titled peptide as a white powder. Amino acid analysis was consistent with the desired structure.

Example 2

SEQUENCE ID NO:8 wherein the Glutamic Acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Proline residue at SEQUENCE position 9 is Pro-NHCH₂CH₃.

The peptide was assembled on a 1% cross-linked Pro-Merrifield resin (2.0 g, 1.0 mmol of Pro) using the same conditions and protecting groups employed in Example 1, with the exception that dinitrophenol group protection was used for the imidazole group of histidine. The peptide-resin obtained (3.45 g) was stirred with ethylamine (20 ml, 0°C) for 6 hours and excess amine was removed in vacuo. The protected peptide resin was extracted with MeOH and precipitated by the addition of a large excess of EtOAc to give 1.36 g of material. The obtained product was treated and deprotected with HF-anisole and crude peptide obtained after this treatment was purified by

- 10 -

gel filtration followed by partition chromatography to yield the homogeneous peptide cited. Amino acid analysis was consistent with the desired structure.

5 Examples 3-7

Using the above described methods with appropriate modifications well known to the skilled in the art, the following peptides are synthesized:

SEQUENCE ID NO:9 wherein the Proline residue
10 at SEQUENCE position 1 is D-Proline, the Tryptophan residues at SEQUENCE positions 4 and 6 are D-Trp, the Tryptophan residue at SEQUENCE position 7 is D-2-ethyl-Trp, and the Methionine residue at SEQUENCE position 8 is Met-NH₂.

15 SEQUENCE ID NO:10 wherein the Tryptophan residue at SEQUENCE position 2 is D-Trp, the Phenylalanine residue at SEQUENCE position 3 is N-methyl-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-isopropyl-Trp, and the Methionine residue at
20 SEQUENCE position 6 is Met-NH₂.

SEQUENCE ID NO:11 wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-methyl-Trp, and the Cysteine residue at SEQUENCE position 7 is Cys-
25 NHCH(CH₂OH)CHOHCH₃ (cyclic disulphide).

SEQUENCE ID NO:12 wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-methyl-Trp, and the Tryptophan residue at SEQUENCE position 8 is Trp-NH₂,
30 (cyclic disulphide).

SEQUENCE ID NO:13 wherein the Tryptophan residue at SEQUENCE position 2 is D-2-methyl-Trp, the Phenylalanine residue at SEQUENCE position 5 is D-Phe, and the Lysine residue at SEQUENCE position 6 is Lys-
35 NH₂.

- 11 -

Although the aforementioned examples of the present invention disclose specific embodiments thereof, it is believed that the substitution of an D-2-alkylTryptophan in bioactive peptide containing at least one Tryptophan residue will yield bioactive peptides providing the advantages and benefits discussed above.

The incorporation of a D-2-alkylTryptophan in bioactive peptides as described above provides a method for prolonging and preserving the activity of such peptides. When analogous bioactive peptides not substituted with an D-2-alkylTryptophan are exposed to various processing conditions and substances, the activity of such peptides may be adversely effected. Sterilizing procedures used in the pharmaceutical industry may expose bioactive compounds to ionizing radiation. Such radiation may effect the formation of reactive radicals. Tryptophan containing peptides are particularly susceptible to attack by such radicals and such attack may render the peptide ineffective, or possibly toxic. Furthermore, various formulating compounds, such as polylactic-polyglycolic acid polymers may contain active, or activated groups which may also attack Tryptophan containing bioactive peptides. The present invention provides a method for protecting a tryptophan containing bioactive peptide from these manufacturing hazards while also increasing the peptides resistance to oxidative degradation after formulation is complete. It is believed that the presence of the alkyl group at the number 2 position of the Tryptophan increases the stability of the pyrrole ring wherein attack by reactive radicals and active or activated groups occurs.

While it is apparent that the invention herein disclosed is well calculated to fulfill the objects above stated, it will be appreciated that numerous embodiments and modification may be devised by

- 12 -

those skilled in the art, and it is intended that the appended claims cover all such modification and embodiments as fall within the true spirit and scope of the present invention.

5

10

15

20

25

30

35

SEQUENCE LISTING

- (1) INFORMATION FOR SEQ ID NO:1
- (i) SEQUENCE CHARACTERISTICS
- 5 (A) LENGTH: 5
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1
- Glu His Trp Ser Tyr
- 1 5
- (2) INFORMATION FOR SEQ ID NO:2
- 15 (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 9
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TYPOLOGY: linear
- 20 (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2
- Phe Cys Tyr Pro Gln Gln Trp Phe Trp
- 1 5
- 25 (3) INFORMATION FOR SEQ ID NO:3
- (i) SEQUENCE CHARACTERISTICS
- 30 (A) LENGTH: 4
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- 35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3
- Leu Arg Pro Gly
- 1

- 14 -

- (4) INFORMATION FOR SEQ ID NO:4
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 4
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4
- Lys Val Cys Trp
- 1
- (5) INFORMATION FOR SEQ ID NO:5
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 4
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5
- Ala Trp Phe Lys
- 1
- (6) INFORMATION FOR SEQ ID NO: 6
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 6
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6
- Pro Gln Gln Trp Phe Trp
- 1 5

- 15 -

(7) INFORMATION FOR SEQ ID NO: 7

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7
- Glu His Trp Ser Tyr Trp Leu Arg Pro Gly
1 5 10

(8) INFORMATION FOR SEQ ID NO: 8

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8
- Glu His Trp Ser Tyr Trp Leu Arg Pro
1 5

(9) INFORMATION FOR SEQ ID NO: 9

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9
- Pro Gln Gln Trp Phe Trp Trp Met
1 5

- 16 -

(10) INFORMATION FOR SEQ ID NO:10

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10
- Arg Trp Phe Trp Leu Met
1 5

(11) INFORMATION FOR SEQ ID NO:11

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 7
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11
- Phe Cys Phe Trp Lys Thr Cys
1 5

(12) INFORMATION FOR SEQ ID NO: 12

- (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 8
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear, and cyclical oxidation product
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12
- Phe Cys Tyr Trp Lys Val Cys Trp
1 5

- 17 -

(13) INFORMATION FOR SEQ ID NO: 13

- 5 (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 6
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

His Trp Ala Trp Phe Lys
1 5

(14) INFORMATION FOR SEQ ID NO: 14

- 15 (i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TYPOLOGY: linear
- 20 (ii) MOLECULE TYPE: Peptide
- (iii) HYPOTHETICAL: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14
- 25 Glu His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

- 18 -

CLAIMS

1. Bioactive peptides containing D-Tryptophan
5 residues wherein at least one D-Tryptophan residue is
replaced with a D-2-alkylTryptophan having a lower
alkyl group substituted at the number 2 position, said
D-2-alkylTryptophan providing increased oxidation
resistance to the peptide while maintaining
10 substantially the same pharmacological effect as
analogous bioactive peptides in which the at least one
Tryptophan residue is not replaced.

2. The bioactive peptides of claim 1 wherein the
lower alkyl group includes 1 to 3 carbons.

15 3. The bioactive peptides of claim 2 wherein the
lower alkyl group is methyl, ethyl, or isopropyl.

4. Bioactive peptides characterized by the
formula A-D-2-alk-Trp-B, wherein alk is a lower alkyl
group;

20 A is SEQUENCE ID NO:1 wherein the Glutamic
acid residue is pyroGlu; D-Phe-Cys-Phe; D-Phe-Cys-Tyr;
SEQUENCE ID NO:6 wherein the Proline residue is D-Pro
and the Tryptophan residues at SEQUENCE positions 4 and
6 are D-Trp; Arg-D-Trp-N-methyl-Phe; or His; and

25 B is SEQUENCE ID NO:3 wherein the Glycine
residue is Gly-NH₂; Leu-Arg-Pro-NHCH₂CH₃;
Lys-Thr-Cys-NHCH(CH₂OH)CHOH(CH₃), SEQUENCE ID NO:4
wherein the Tryptophan residue is Trp-NH₂; Met-NH₂;
Leu-Met-NH₂ or SEQUENCE ID NO:5 wherein the
30 Phenylalanine residue is D-Phe and the Lysine residue
is Lys-NH₂ provided that:

when A is SEQUENCE ID NO:1 wherein the
Glutamic acid residue is pyroGlu, B is SEQUENCE ID NO:3
wherein the Glycine residue is GlyNH₂ or
35 Leu-Arg-Pro-NHCH₂CH₃;

- 19 -

when A is D-Phe-Cys-Phe, B is Lys-Thr-Cys-NHCH(CH₂OH)CHOH(CH₃) wherein the cysteine units are bound to each other by a cyclic disulphide;

when A is D-Phe-Cys-Tyr, B is SEQUENCE ID NO:4 wherein the Tryptophan residue is Trp-NH₂ and the cysteine units are bound to each other by a cyclic disulfide;

when A is SEQUENCE ID NO:6 wherein the Proline residue is D-Pro and the Tryptophan residues at SEQUENCE positions 4 and 6 are D-Trp, B is Met-NH₂;

when A is Arg-D-Trp-N-methyl-Phe, B is Leu-Met-NH₂; and

when A is His, B is SEQUENCE ID NO:5 wherein the Phenylalanine residue is D-Phe and the Lysine residue is Lys-NH₂; wherein said peptides provide increased resistance to ionized sterilization, reactive radicals, and oxidation while maintaining a similar pharmacological effect as compared to analogous D-Tryptophan containing peptides not containing at least one D-2-alkylTryptophan.

5. The bioactive peptides of claim 4 wherein the lower alkyl group includes 1 to 3 carbon atoms.

6. The bioactive peptides of claim 5 wherein the alkyl group is methyl, ethyl, or isopropyl.

7. The bioactive peptides according to claim 4 wherein said peptide is SEQUENCE ID NO: 7 wherein the Glutamic acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Glycine residue at SEQUENCE position 10 is Gly-NH₂; SEQUENCE ID NO:8 wherein the Glutamic Acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Proline residue at SEQUENCE position 9 is Pro-NHCH₂CH₃; SEQUENCE ID NO:9 wherein the Proline residue at SEQUENCE position 1 is D-Proline, the Tryptophan residues at SEQUENCE positions 4 and 6

- 20 -

are D-Trp, the Tryptophan residue at SEQUENCE position 7 is D-2-ethyl-Trp, and the Methionine residue at SEQUENCE position 8 is Met-NH₂; SEQUENCE ID NO:10 wherein the Tryptophan residue at SEQUENCE position 2 is D-Trp, the Phenylalanine residue at SEQUENCE position 3 is N-methyl-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-isopropyl-Trp, and the Methionine residue at SEQUENCE position 6 is Met-NH₂; SEQUENCE ID NO:13 wherein the Tryptophan residue at SEQUENCE position 2 is D-2-methyl-Trp, the Phenylalanine residue at SEQUENCE position 5 is D-Phe, and the Lysine residue at SEQUENCE position 6 is Lys-NH₂.

8. The bioactive peptides according to claim 4 wherein said peptide is SEQUENCE ID NO:11 wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-methyl-Trp, and the Cysteine residue at SEQUENCE position 7 is Cys-NHCH(CH₂OH)CHOHCH₃, and wherein the cysteine units are bound to each other by means of a cyclic disulphide bond; or SEQUENCE ID NO: 12 wherein the Phenylalanine residue at SEQUENCE position 1 is D-Phe, the Tryptophan residue at SEQUENCE position 4 is D-2-methyl-Trp, and the Tryptophan residue at SEQUENCE position 8 is Trp-NH₂, wherein the cysteine units are bound to each other by means of a cyclic disulphide bond.

9. Bioactive peptides containing D-2-alkylTryptophan residues wherein said peptides are SEQUENCE ID NO:7 wherein the Glutamic acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the Glycine residue at SEQUENCE position 10 is Gly-NH₂; SEQUENCE ID NO:8 wherein the Glutamic Acid residue at SEQUENCE position 1 is Pyro-Glu, the Tryptophan residue at SEQUENCE position 6 is D-2-methyl-Trp, and the

- 21 -

Proline residue at SEQUENCE position 9 is Pro-NHCH₂CH₃;
SEQUENCE ID NO:9 wherein the Proline residue at
SEQUENCE position 1 is D-Proline, the Tryptophan
residues at SEQUENCE positions 4 and 6 are D-Trp, the
5 Tryptophan residue at SEQUENCE position 7 is D-2-ethyl-
Trp, and the Methionine residue at SEQUENCE position 8
is Met-NH₂; SEQUENCE ID NO:10 wherein the Tryptophan
residue at SEQUENCE position 2 is D-Trp, the
Phenylalanine residue at SEQUENCE position 3 is N-
10 methyl-Phe, the Tryptophan residue at SEQUENCE position
4 is D-2-isopropyl-Trp, and the Methionine residue at
SEQUENCE position 6 is Met-NH₂; SEQUENCE ID NO:11
wherein the Phenylalanine residue at SEQUENCE position
1 is D-Phe, the Tryptophan residue at SEQUENCE position
15 4 is D-2-methyl-Trp, and the Cysteine residue at
SEQUENCE position 7 is Cys-NHCH(CH₂OH)CHOHCH₃, and
wherein the cysteine units are bound to each other by
means of a cyclic disulphide bond; SEQUENCE ID NO: 12
wherein the Phenylalanine residue at SEQUENCE position
20 1 is D-Phe, the Tryptophan residue at SEQUENCE position
4 is D-2-methyl-Trp, and the Tryptophan residue at
SEQUENCE position 8 is Trp-NH₂, wherein the cysteine
units are bound to each other by means of a cyclic
disulphide bond; and SEQUENCE ID NO:13 wherein the
25 Tryptophan residue at SEQUENCE position 2 is D-2-
methyl-Trp, the Phenylalanine residue at SEQUENCE
position 5 is D-Phe, and the Lysine residue at SEQUENCE
position 6 is Lys-NH₂.

10. A method for prolonging the pharmacological
30 activity of a bioactive peptide containing one or more
D-Tryptophan residues which comprises replacing at
least one D-Tryptophan residue of said peptide with a
D-2-alkylTryptophan residue so as to increase said
peptides resistance to degradation by oxygen, reactive
35 radicals and other reactive groups while maintaining a
similar biological effect as compared to analogous

- 22 -

bioactive peptides wherein the Tryptophan residue is not so replaced.

11. A method for preserving the pharmacological effectiveness of a bioactive peptide containing one or
5 more Tryptophan residues which is to be sterilized or exposed to active or activated groups during manufacture which comprises replacing at least one Tryptophan residue of said peptide with a D-2-alkylTryptophan residue so as to increase the
10 resistance of said peptide to the effects of sterilization or exposure to active or activated groups as compared to analogous bioactive peptides wherein the at least one Tryptophan residue is not so replaced.

12. The method of claim 11 wherein the bioactive
15 peptide is sterilized by exposure to ionizing radiation.

13. The method of claim 11 wherein the peptide is exposed to the active or activated groups present in controlled delivery substances based on polylactic-
20 polyglycolic acid polymers.

25

30

35

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/00727

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 07 K 7/20, 7/26, 7/06, A 61 K 37/24, 37/43		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵	C 07 K, A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	The New England Journal of Medicine, 29 March 1990, R.M. Silver et al.: "Scleroderma, fasciitis, and eosinophilia associated with the ingestion of tryptophan", pages 874-881 see the whole document cited in the application ---	1-3
A	US, A, 3316260 (TSUNG-YING SHEN) 25 April 1967 see column 2, lines 65-72; column 3, lines 26-46; examples 1-55; claims 1-4 ---	1-3
A	Chem. Pharm. Bull., volume 27, no. 8, 1979, Y. Yabe et al.: "Synthesis and biological activity of LH-RH Analogs substituted by alkyltryptophans at position 3", pages 1907-1911 see the whole document ---	1-3, 10, 11
A	Endocrine Reviews, volume 7, no. 1, 1986, The Endocrine Society (US), M.J. Karten et al.: "Gonadotropin-releasing hormone ./.	4, 7, 9
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
4th June 1991	17 SEP 1991	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	<p>analog design. Structure-function studies toward the development of agonists and antagonists: Rationale and perspective", pages 44-66 see page 46</p> <p>---</p>	
A	<p>EP, A, 0203031 (THE ADMINISTRATORS OF THE TULANE UNIVERSITY EDUCATIONAL FUND) 26 November 1986 see the whole document</p> <p>---</p>	4,8,9
A	<p>Proc. Natl. Acad. Sci. USA, volume 83, 1986, Y. Torrens et al.: "Substance P receptors in primary cultures of cortical astrocytes from the mouse", pages 9216-9220 see the whole document</p> <p>---</p>	4,7,9
A	<p>EP, A, 0083864 (BECKMAN INSTRUMENTS) 20 July 1983 see the whole document</p> <p>-----</p>	4,7,9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9100727
SA 46621

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 19/08/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 3316260		BE-A- 688765	31-03-67
		CH-A- 468378	
		CH-A- 476724	15-08-69
		CH-A- 487140	15-03-70
		DE-A- 1543840	21-05-70
		DE-A, B, C 1620222	06-04-72
		FR-M- 5110	29-05-67
		FR-M- 6717	17-02-69
		FR-M- 6041	20-05-68
		FR-A- 1458096	
		FR-A- 1497423	
		FR-A- 1602629	04-01-71
		GB-A- 1080470	
		GB-A- 1140803	
		GB-A- 1169014	29-10-69
		NL-A- 6415318	02-07-65
		NL-A- 6614056	25-04-67
		NL-A- 6615029	25-04-67
		US-A- 3419555	
		US-A- 3470189	30-09-69
EP-A- 0203031	26-11-86	US-A- 4650787	17-03-87
		US-A- 4725577	16-02-88
		AU-B- 600895	30-08-90
		AU-A- 5633886	30-10-86
		JP-A- 61293997	24-12-86
EP-A- 0083864	20-07-83	US-A- 4410512	18-10-83
		US-A- 4410513	18-10-83
		US-A- 4411890	25-10-83
		AU-B- 549053	09-01-86
		CA-A- 1242435	27-09-88
		WO-A- 8302272	07-07-83

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)